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Development of a viability standard curve for microencapsulated probiotic bacteria using confocal microscopy and image analysis software

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ABSTRACT

Microencapsulation is proposed to protect probiotic strains from food processing procedures and to maintain probiotic viability. Little research has described the in situ viability of microencapsulated probiotics. This study successfully developed a real-time viability standard curve for microencapsulated bacteria using confocal microscopy, fluorescent dyes and image analysis software.

1. Introduction

Probiotic bacteria are reported to convey health benefits to hosts and are included in a number of food products, such as yoghurt and cheese (Davis, 2014; Parvez et al., 2006). The viability of probiotic bacteria is considered an important characteristic in the health-conveying properties (Fuller, 1992). The World Health Organisation has defined probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). However, the viability of such bacteria is significantly reduced during food processing procedures and storage (Kailasapathy, 2002). Consumers are reported to receive less than the recommended amount of viable probiotics at the time of purchase and consumption (Kosin and Rakshit, 2006). Microencapsulation has been proposed to 'protect' probiotics from manufacturing and processing procedures and to assist in maintaining probiotic viability during food storage (Kailasapathy, 2002). Furthermore, microencapsulation has been postulated to protect probiotics during transit through the acidic conditions of the gastrointestinal tract (Kailasapathy, 2008).

One of the most common methods to enumerate microencapsulated bacteria is to release the bacteria from the capsules (usually by homogenisation) and to grow the bacteria on suitable media (Annan et al., 2007; Champagne et al., 2010; Ding and Shah, 2008; Zou et al., 2011). The numerous steps to rehydrate and plate microencapsulated probiotics can underestimate the colony forming units (Champagne et al., 2011) and do not provide real-time live and dead cell information. A number of culture-independent methods can be used to enumerate probiotic bacteria including fluorescence imaging, real-time PCR and cell sorting techniques such as 16S rRNA quantification, MALDI-TOF mass spectrometry and flow cytometry/FACS (Davis, 2014). Fluorescent stains, microscopy instruments and image analysis software provide the tools to evaluate the real-time viability of bacteria cells in a capsular environment without releasing the bacteria cells. It has been established that the viability of microencapsulated probiotics is comparatively higher than free bacteria in conditions simulating the gastrointestinal tract (Mandal et al., 2006) providing support that microencapsulation provides a suitable vehicle to protect probiotics. However, further research is required to determine the behaviour of probiotics whilst encapsulated and to explore methods that determine the real-time viability of microencapsulated probiotics. The real-time viability of microencapsulated probiotic strains using flow cytometry has been described. Flow cytometry is ideal to gain information about multiple parameters for a large number at one time (Tracey et al., 2010). The use of various protectants was shown to improve the survival of probiotic strains in simulated gastrointestinal environments using flow cytometry (Chen et al., 2012). Confocal microscopy has also been used to investigate microcapsules. The integrity of microcapsules during storage in apple juice has been examined using confocal microscopy (Ying et al., 2012). The structure and thickness of alginate coating on gelatine microspheres were described using fluorescein labelled sodium alginate

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and confocal microscopy (Annan et al., 2008). Advances in fluorescence microscopy technologies have provided another tool to measure the real-time microencapsulated probiotic viability within the capsule environment without releasing the internal bacteria cells, as well as provide a 3D perspective of cell distribution within the capsules.

In this study, two probiotic strains (*Lactobacillus acidophilus* LAFTI® L10 and *Bifidobacterium lactis* HN019) and a pathogenic strain (*Streptococcus pyogenes*) were stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen, Mulgrave, Australia). The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit employs two nucleic acid fluorescent stains, SYTO® 9 and propidium iodide (PI), to distinguish between live and dead bacteria cells, respectively. The strains were individually microencapsulated in polymers of alginate and chitosan. The cell distribution of the microencapsulated bacteria was determined using laser-scanning confocal microscopy (LSCM). A viability standard curve for each microencapsulated bacteria strain was developed using the 3D images (z-stacks) obtained from LSCM and Bitplane Imaris image analysis software.

2. Materials and methods

2.1. Bacterial strains and media

Two strains of probiotic bacteria and a pathogen strain were studied: the probiotics *L. acidophilus* LAFTI® L10 (DSM Nutritional Products; Sydney, Australia) and *B. lactis* HN019 (DR10™) (Fonterra Co-operative Group, New Zealand), and the pathogenic strain, *S. pyogenes* (ATCC 196155). The probiotic bacterial strains were grown in de Man, Rogosa and Sharpe (MRS) broth (Oxoid) at 37 °C for 24 h under anaerobic conditions using an anaerobic chamber (Oxoid) and Anaero*Gen*™ sachet (Oxoid). The *S. pyogenes* cultures were grown aerobically at 37 °C for 24 h in Brain Heart Infusion (BHI) broth (Oxoid).

2.2. Microencapsulation materials

Alginate solution (1.8%) was prepared by dissolving 9 g of alginic acid sodium salt (Sigma-Aldrich) in 450 mL of milli Q water using a magnetic stirrer and heated to 50 °C. The volume was adjusted to 500 mL once the alginic acid was completely dissolved. The alginate solution was filtered using a sterile Sartopure PP2 capsule (0.65 μM) (Sartorius) and the Millipore easy-load pump (Masterflex®) and then a 0.22 μm Steritop™ Filter Unit (Millipore). The alginate solution was stored at room temperature.

The cation solution contained chitosan from crab shell (Sigma-Aldrich) at a concentration of 0.1% w/v in 0.1 M calcium chloride (Sigma-Aldrich). The chitosan was initially dissolved in 20% glacial acetic acid (Ajax Finechem) and $CaCl_2$ (0.1 M, pH 6.2) was later added making a final volume of 1 L. The cation solution was stored at 4 °C. Brij solution contained zinc chloride (0.2 mM) (Ajax Chemicals) and Brij (150 μL) (Sigma-Aldrich) at a final volume of 100 mL, and stored at room temperature.

2.3. Staining of bacteria with LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit (Invitrogen)

The *L. acidophilus* LAFTI® L10, *B. lactis* HN019 (DR10™) and *S. pyogenes* strains were cultured for 24 h. The cultures were enumerated by absorbance measurements at 600 nm using the Bio-Rad Benchmark Plus Microplate Spectrophotometer (Bio-Rad) and standard curves developed for each strain. The bacteria cells were harvested by centrifugation using the Universal 32 R centrifuge (HD Scientific Supplies Pty. Ltd.) at 3864 *g* for 10 min (37 °C). The supernatant was decanted and the bacteria cells were each initially resuspended in the respective media (MRS or BHI broth) making a final concentration of 1.0×10^{10} CFU mL^{-1}. From this suspension, 2 × 2.5 mL aliquots were centrifuged, the supernatant was discarded and the first aliquot was resuspended in 20 mL of sterile NaCl (0.85%) (Sigma-Aldrich) solution to give a live cell suspension. The second aliquot was resuspended in 20 mL of ethanol (100%) to give a dead cell suspension. All bacterial cultures were incubated at room temperature for 1 h and mixed by vortex briefly (approximately 5 s) every 20 min. The bacteria cultures were centrifuged, the supernatant was removed, and the pellets were washed with 20 mL of NaCl (0.85%) and re-centrifuged. The supernatant was removed and the pellets were resuspended in 2.5 mL of NaCl (0.85%). Different viability concentrations were prepared by mixing the live and dead cell suspensions. Concentrations of 0, 10, 50, 90 and 100% live cells were prepared in 1 mL volumes.

A 1:1 ratio of SYTO® 9 and propidium iodide (PI) stains from the LIVE/DEAD® *Bac*Light™ Bacterial Viability and Counting Kit (Invitrogen) was prepared; the stains were mixed by pipetting. The 1 mL bacteria aliquots of different viabilities were stained with 2 μL of the SYTO® 9 and PI mix and the bacteria and dye suspension was incubated for 10 min at room temperature in the dark prior to microencapsulation and microscopy.

2.4. Microencapsulation of bacteria

A 200 μL aliquot of Brij solution and alginate solution (1.8%) was added to the 1 mL bacteria/fluorescent dye suspensions making a final volume of 10 mL (final cell concentration approximately

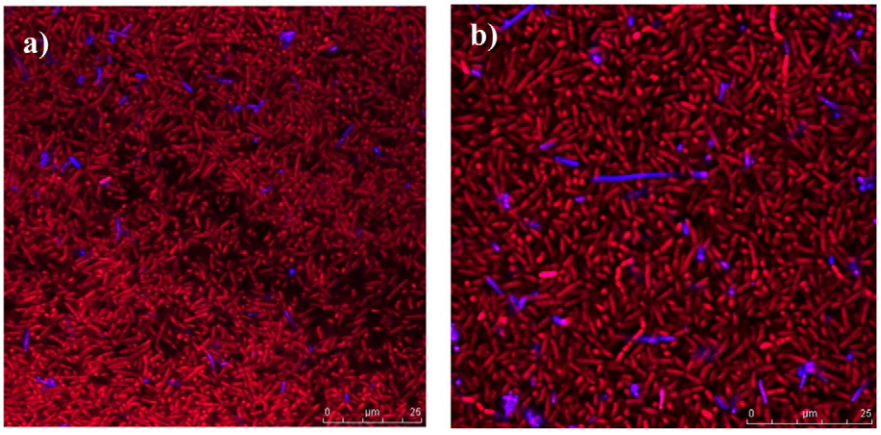

Fig. 1. LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen) treated non-microencapsulated probiotics a) *Lactobacillus acidophilus* LAFTI® L10 and b) *Bifidobacterium lactis* HN019 (DR10™). Live cells (expressing SYTO® 9) are shown in red and dead cells (expressing propidium iodide) are shown in blue. Scale bar = 25 μM, n = 3.